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Title: Effects of Acute Stress and Exercise on Subsequent Seawater Adaptation and Cortisol Dynamics in Juvenile Steelhead Trout (*Oncorhynchus mykiss*).

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Carl B. Schreck

The present study investigated the effects of stress and exercise on seawater (SW) adaptation and cortisol dynamics in juvenile steelhead (*Oncorhynchus mykiss*). To examine the effects of stress, fish acclimated to freshwater (FW) were subjected for 3 hours to confinement stress in FW, and subsequently SW (25 ppt) was introduced to all tanks. Fish were sampled immediately after the stress treatment, and 1, 7, and 14 days after introduction of SW. Electrolytes, cortisol, glucose and lactate followed the typical pattern that we expected after stress treatment in FW. Fish regained osmotic balance within 24 hours. Glucose concentrations were increasing throughout the experiment and lactate levels stayed elevated during the time spent in SW. IGF-1 did not show an immediate response to stress but after transfer to SW we detected significantly higher concentrations for control fish at days 1 and 14. The differences in IGF-1 levels between stressed and control fish are not reflected in

SW adaptability but positive correlations between IGF-1 and electrolyte levels in control fish may indicate its role for osmoregulation. Confinement stress did not impair feed intake subsequently in SW, but our results suggest that feed intake was suppressed by the change of the media from FW to SW.

The second study investigated the effects of exercise treatment in FW on SW adaptation and cortisol dynamics in juvenile steelhead. Plasma cortisol and *in vitro* cortisol secretion by interrenal cells after a 24 hr SW challenge test were neither affected by moderate exercise nor by water temperature (13 °C, 21 °C), however, plasma osmolality was lower in exercised fish compared to unexercised fish. Half-life ($T_{1/2}$) of ^{3}H-cortisol was shorter in fish exposed to exercise whereas metabolic clearance rate (MCR) did not respond to exercise treatment. Uptake and retention of corticosteroids in liver and gall bladder were enhanced in exercised fish, and retention of corticosteroids in muscle tissue was longer in unexercised fish. Our findings suggest that exercise likely decreases stress levels in fish and improves the adaptation to seawater (SW) in juvenile steelhead.

Effects of Acute Stress and Exercise on Subsequent Seawater Adaptation and Cortisol Dynamics in Juvenile Steelhead Trout (*Oncorhynchus mykiss*).

by

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APPROVED:

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Anja M. Liebert, Author

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CONTRIBUTION OF AUTHORS

Dr. Schreck was involved in the research design, analysis and editing of both manuscripts. Dr. Feist was involved in research design, analysis and editing of the 2nd manuscript.

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INTRODUCTION

Cortisol and growth hormone (GH), that stimulates the liver to produce insulin-like growth factor-1 (IGF-1), are the main regulators of hydromineral balance in euryhaline teleosts during seawater (SW) adaptation (McCormick 1995). Cortisol and IGF-1 increase gill Na^+, K^+ ATPase activity and therefore can enhance SW tolerance of fish (Madsen and Bern 1993, Madsen et al. 1995, McCormick 1995, 1996). Cortisol is also a primary messenger of the stress response and is released from interrenal tissue in the head kidney shortly after exposure to an acute stressor. Stress can influence osmoregulation directly or indirectly. It disturbs osmoregulation by increasing the oxygen demand consequent to high catecholamine levels and increasing the effective respiration surface area due to increased blood flow (Nilsson 1986). An indirect effect of stress is delayed or reversed smoltification (Schreck et al. 1985, Patiño et al. 1986a) that could either result in reduced SW survival or delayed SW entry. The interaction of IGF-I with the interrenal axis during SW adaptation after exposure to stressors and the implication of the metabolic status of the fish are not well understood.

Cortisol has also been implicated as key hormone in adaptation to exercise (Lassourd et al. 1996). An increase of cortisol levels after exercise has been reported in mammals (Cashmore et al. 1977, Dybdal et al. 1980, Lassourd et al. 1996). Exercise increases metabolic costs for an animal; therefore, the ability of fish to adapt to a marine environment could be reduced if they are in an energy deficit.

The goal of this study is to determine the effects of stress and exercise in fresh water (FW) adapted juvenile steelhead on subsequent SW adaptation. This thesis is organized into four chapters. The introduction follow two chapters that are presented in manuscript form, chapter IV is a general conclusion drawn from the results of chapters II and III. Chapter II investigates the effects of acute stress on feeding behavior, and the dynamics of cortisol and IGF-1 in SW in juvenile steelhead. Chapter III investigates the influences of exercise and water temperature on following SW adaptation, and on corticosteroid dynamics in juvenile steelhead.

EFFECTS OF ACUTE STRESS ON OSMOREGULATION, FEED INTAKE, IGF-1 AND CORTISOL IN YEARLING STEELHEAD TROUT (*ONCORHYNCHUS MYKISS*) DURING SEAWATER ACCLIMATION

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Abstract

Juvenile steelhead (*Oncorhynchus mykiss*) acclimated to freshwater (FW) were subjected for 3 hours to confinement stress in FW, and subsequently seawater (SW, 25 ppt) was introduced to all tanks. Fish were sampled immediately after the stress treatment, and 1, 7, and 14 days after introduction of SW. Electrolytes, cortisol, glucose and lactate showed the typical stress response expected after stress treatment in FW. Fish regained osmotic balance within 24 hours. Glucose concentrations were increasing throughout the experiment and lactate levels stayed elevated during the time spent in SW. IGF-1 did not show an immediate response to stress but after transfer to SW we detected significantly higher concentrations for control fish at days 1 and 14. The differences in IGF-1 levels between stressed and control fish are not reflected in SW adaptability but positive correlations between IGF-1 and electrolyte levels in control fish may indicate its role for osmoregulation. Confinement stress did not impair feed intake subsequently in SW, but our results suggest that feed intake was suppressed by the change of the media from FW to SW.

Introduction

Stress increases the osmotic imbalance when FW adapted salmonids are exposed to SW (Redding and Schreck, 1983). Stress in teleosts is characterized by the immediate release of catecholamines and cortisol; both hormones are concerned with energy reallocation from anabolic activities such as growth toward activities to restore homeostasis (Wendelaar Bonga, 1997).

Cortisol and GH, which among the many functions stimulates the liver to produce insulin-like growth factor-1 (IGF-1), are the main regulators of

hydromineral balance in euryhaline teleosts during SW adaptation (McCormick, 1995). Cortisol and IGF-1 increase gill Na^+, K^+ ATPase activity and therefore can enhance SW tolerance of fish (Madsen and Bern, 1993, Madsen et al., 1995, McCormick, 1995, 1996). Most of the IGF-1 is bound to IGF-binding proteins (IGF-BP) that regulate the interaction of the ligand with receptors and hence affect tissue availability (Jones and Clemmons, 1995, Kelley et al., 2001); only 0.3% of the total amount of IGF-1 is free in the plasma (Shimizu et al., 1999). During catabolic states such as food deprivation and stress, IGF-BP's (< 30 kDa) are up-regulated to inhibit energy expensive growth processes (Kelley et al., 2001). In Atlantic salmon (*Salmo salar*) elevated IGF-1 levels were observed after repeated acute stress (McCormick et al., 1998).

Feed intake is reduced after acute stress (Pickering et al., 1982, McCormick et al., 1998), and reduced appetite has been attributed to elevated cortisol levels (Gregory and Wood, 1999). Abrupt transfer of juvenile salmonids from FW to SW also results in suppression of feed intake (Usher et al., 1991, Jørgensen and Jobling, 1994, Arnesen et al., 1998, 2003). The relationship between feed intake and IGF-1 in salmonids has not clearly been established; Moriyama et al. (1994) and Aas-Hansen et al. (2003) found that IGF-1 was positively correlated to feed intake whereas McCormick et al. (1998) found no evidence of a relationship between these variables.

The objective of our study was to determine the effect of acute stress in FW-adapted yearling steelhead on feeding behavior and the dynamics of cortisol and IGF-1 in SW and correlate it to SW adaptation.

Materials and methods

Fish

The experiment was carried out at Oregon State University's Hatfield Marine Science Center in Newport, Oregon. Yearling steelhead from the Alsea River stock (Oregon Department of Fish and Wildlife) were transported to Newport on February 18, 2003. This stock of fish is thought to smolt around early May (Wagner, 1974) Fish were transferred into 1 m diameter circular tanks with 12-13° C aerated, pathogen free, flow-through, de-chlorinated well water. The fish were fed twice daily by hand in excess with dry feed (Bio Dry 1000, Bio-Oregon, Warrenton, OR).

Experimental design

Fish were randomly assigned to six tanks, 35 fish per tank; all treatment groups were triplicated. Fish were allowed to acclimate in fresh water until they fed well. On March 7, 2003, a stressor was applied to the fish in the corresponding tanks. The stressor consisted of netting the fish into a perforated 18-liter bucket, holding them up into the air for 30 s, and subsequently keeping the fish confined for three hours in the perforated bucket immersed in their tank. The fish were released back into their respective tank. The control group did not experience the stress. Immediately after the stress treatment SW was introduced into all tanks yielding a final concentration of approximately 25 ppt after 120 min by mixing FW and SW (4 l min^{-1}). This salinity was chosen after a preliminary experiment demonstrated that full strength SW was lethal to some of the fish at this stage. 5 fish per tank were sampled immediately after the stress treatment but

before SW exposure, and 10 fish per tank were sampled on days 1, 7 and 14 after the initiation of SW.

Sampling procedures

Fish were fed twice per day in excess; on sampling dates fish were fed in excess a labeled feed in the morning approximately 0.3 hr before sampling. Fish were netted from each tank and killed with a lethal dose of tricaine methansulfonate (MS 222, 200 mg/l, buffered with $NaHCO_3$). Fish were weighed (to 0.1 g) and measured for fork length (to 1 mm). Blood samples were taken from the caudal peduncle using heparinized vacutainers (Becton Dickinson). Blood was centrifuged for 7 min, plasma removed, frozen on dry ice and stored at –80 °C until further analysis. In a few cases equal amounts of plasma from several fish of the same tank were pooled for analysis. A small sample of dorsal musculature was taken to determine muscle water content. These samples were dried at 60 °C in pre-weighed vials until vials reached a constant weight.

Determination of feed intake

The feed had previously been ground into powder and repelleted. A small batch of the ground feed was mixed with small lead glass beads (ballotini size 9, Jencons Ltd, Leighton Buzzard, U.K., 3% of feed weight) before repelletization for the estimation of feed intake by X-radiography (Jobling et al. 1995). Known amounts of the labeled feed were X-rayed to establish the relationship between number of ballotinis and weight of the feed: weight = 0.0092 * number ballotini + 0.0053, $R^2 = 0.9543$. To estimate feed intake individually, carcasses were X-rayed (Faxitron 804 cabinet X-ray machine, AGFA Structurix D7 film, 150 s exposure time, 35 kV), and the number of ballotinis in the gastrointestinal tract of each fish

was counted. The weight of the consumed feed was calculated with the equation shown above, and subtracted from the bodyweight before feed intake was expressed in % body weight.

Analyses

The percent muscle water was calculated as the weight difference between fresh tissue and dried tissue. Fish condition factor (K) was calculated according to the formula: K = weight [g] / (length [mm]) ^{3} x 10^5. The mass term in the equation was corrected for food weight in individual fish. Plasma cortisol levels were determined by radioimmunoassay (RIA) after Foster and Dunn (1974), modified by Redding et al. (1984). All fish having less cortisol than 3.9 ng/ml (lowest standard) were given this value. Concentrations of unbound plasma IGF-I were determined with a commercially available Fish IGF-1 RIA kit (GroPep Ltd., Adelaide, Australia) using acid-ethanol extracted plasma. IGF-1 parallelism was checked for steelhead plasma to validate the assay. Plasma osmolality was measured with a vapor pressure osmometer. Plasma concentrations of sodium, potassium, chloride, calcium, magnesium, glucose and lactate were measured with a Nova Biomedical CCR analyzer (Waltham, MA).

Statistical analyses

Statistical tests were performed with S-Plus 6.1. Differences between replicate tanks within sampling date and fish type, between the fish types within sampling date, and between sampling dates within the fish type were analyzed using one-way analysis of variance (one-way ANOVA), followed by a Tukey-Kramer test if there was a significant effect. If replicate tanks within the treatment were not different, tanks were pooled. If differences between replicates occurred, these tanks were compared separately from pooled tanks with the other

treatment. To help identify possible tank effects we applied an ANOVA model that included the effects of sampling date and treatment and their interactions, and the effects of replicates nested within treatment. A chi-square test of independence (Pearson) was applied to investigate differences in the proportions of non-feeding fish between the treatments. Correlations between variables were examined with a linear regression model, separately for each treatment. Proportional data of percent muscle water content and feed intake were arc-sin squared transformed. Cortisol data were transformed to logarithmic values.

Results

Fish size and condition factor

Average fish length was 179 ± 1.23 mm and fish weighed on average 60 ± 1.14 g over the course of the experiment. Fish length and weight were not different between sampling dates. Condition factor varied between 1.0 and 1.1 and was not different between controls and stressed fish (data not shown).

Feed intake

Throughout the experiment feed intake increased in both groups. Stressed fish fed significantly more at two weeks in SW ($p < 0.005$) compared to day 1 or to two of the control tanks at day 14 (Fig. II.1); one of the control tanks had significantly higher feed intake than the two other tanks. In the nested ANOVA model we observed neither tank nor treatment effects for feed intake. The proportion of non-feeders decreased over the course of the experiment from 57% to 16% in stressed fish and from 47% to 33% in controls. On day 14 the proportion of non-feeders was significantly higher in controls compared to stressed fish. We found some evidence that feed intake could be correlated with

osmoregulatory capability. At day 1 feed intake in controls was negatively correlated to magnesium levels. At day 14 we found a negative correlation between feed intake and chloride levels in both treatments. Sodium was negatively correlated to feed intake in stressed fish at day 14. A significant positive correlation between lactate levels and feed intake was noted at days 7 and 14 in stressed fish, and at day 7 in controls. For control fish we found a positive relationship between feed intake and glucose on day 7. A negative correlation between feed intake and cortisol was observed in control fish at day 7.

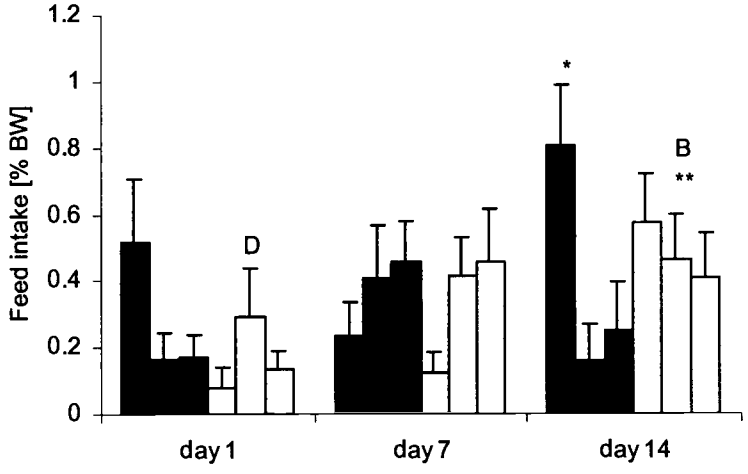
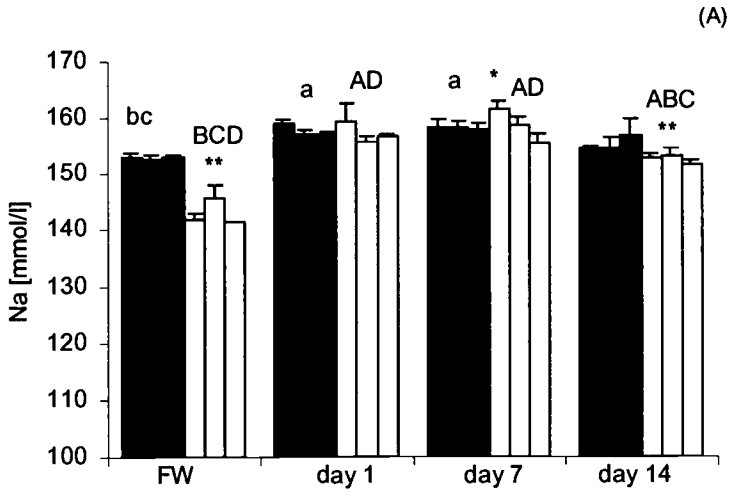


Fig. II.1. Mean (± SE) percent food intake of yearling steelhead 1, 7, and 14 days after introduction of SW (25 ppt). Grey bars show values for controls and white bars for stressed fish, (n = 10). *, significantly different ($p<0.05$) from other tanks within replicate; **, significantly different from pooled controls at same sampling date. Within the same fish type letters indicate significant differences between the different sampling dates, a: different from FW, b: different from day 1, c: different from day 7, d: different from day 14 (symbols and letters refer to all bars within replicate that are not significantly different from each other); capital letters are used for stressed fish and lower case letters for controls.

Osmoregulatory parameters

The confinement stressor caused a highly significant decrease in the plasma concentration of sodium and chloride immediately after the stressor was applied (Fig. II.2 A, B). Potassium, calcium and magnesium concentrations did not change significantly (Fig. II.2 C, II.3 A, B). All electrolytes except potassium and calcium showed a significant increase in both fish types one day after SW transfer. Calcium levels of control fish were significantly higher than for stressed fish. At day 7, sodium, magnesium and potassium levels were similar to day 1. Control fish showed a significant decline in chloride and calcium concentrations. Potassium was significantly higher in controls compared to stressed fish. After two weeks sodium, magnesium and calcium levels of stressed fish declined significantly, whereas potassium levels increased. Chloride of control fish also showed a significant increase and was significantly higher than in stressed fish (Fig. II.2 B). Generally, values for all electrolytes were in the same range as for control fish before SW introduction.



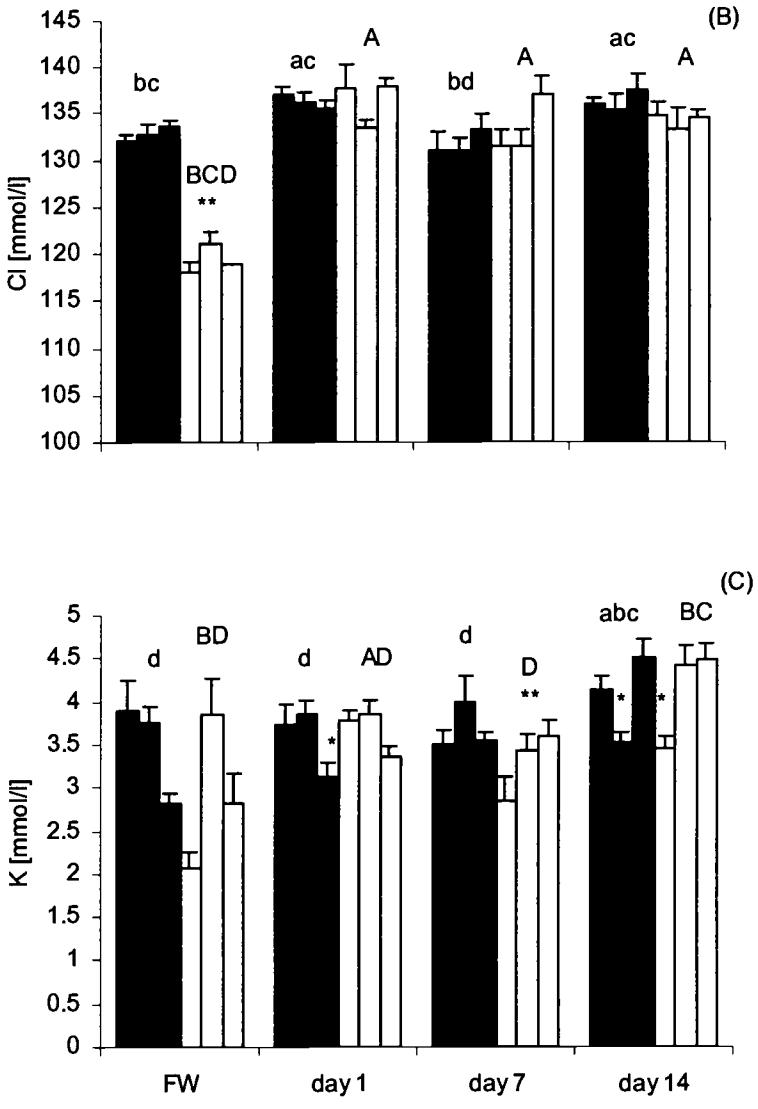


Fig. II.2. Mean (± SE) plasma sodium (A), chloride (B) and potassium (C) concentrations of yearling steelhead immediately after acute stress in FW, and 1, 7, and 14 days after introduction of SW (25 ppt). Grey bars show values for controls and white bars for stressed fish, (n = 8-10, FW: n=5). Indications for significance as shown in Fig. 1.

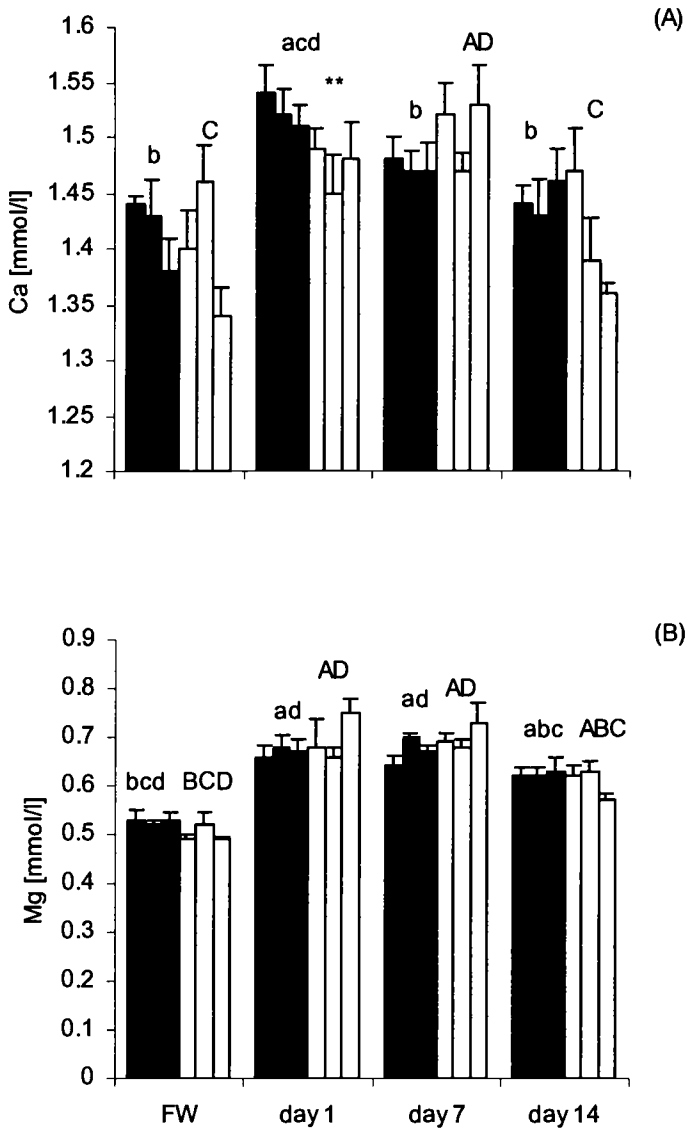


Fig. II.3. Mean (± SE) plasma calcium (A) and magnesium (B) concentrations of yearling steelhead immediately after acute stress in FW, and 1, 7, and 14 days after introduction of SW (25 ppt). Grey bars show values for controls and white bars for stressed fish, (n = 8-10, FW: n=5). Indications for significance as shown in Fig. 1.

For plasma osmolality we did not detect any differences between the two groups but a significant increase over time for both fish types (Fig. II.4). The lack of plasma for day 1 did not allow us to assess osmolality for that day.

Disagreement between replicates was observed for sodium at day 7 in controls,

for potassium at day 1 and 14 in controls, and day 14 in stressed fish, and for osmolality on day 7 in stressed fish. The nested ANOVA model suggested the presence of tank effects for osmolality and potassium but not for sodium. There was no difference in muscle water contents between stressed and control fish at any time. Control fish had significantly lower muscle water content at day 1 compared to other dates (Fig. II.5).

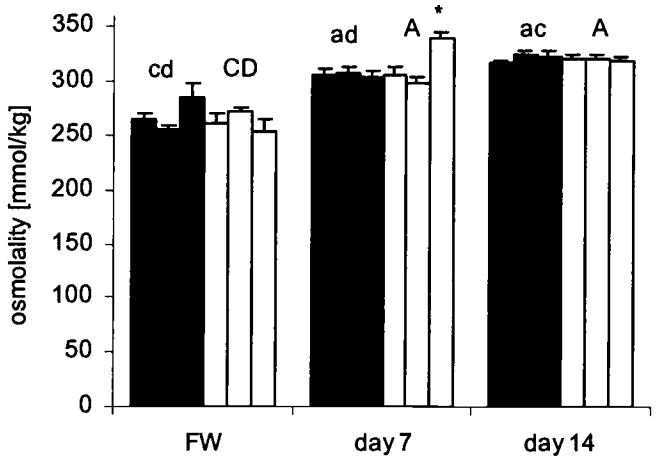


Fig.II. 4. Mean (± SE) plasma osmolality concentrations of yearling steelhead immediately after acute stress in FW, and 7, and 14 days after introduction of SW (25 ppt). Grey bars show values for controls and white bars for stressed fish, (n = 8-10, FW: n=5). Indications for significance as shown in Fig. 1.

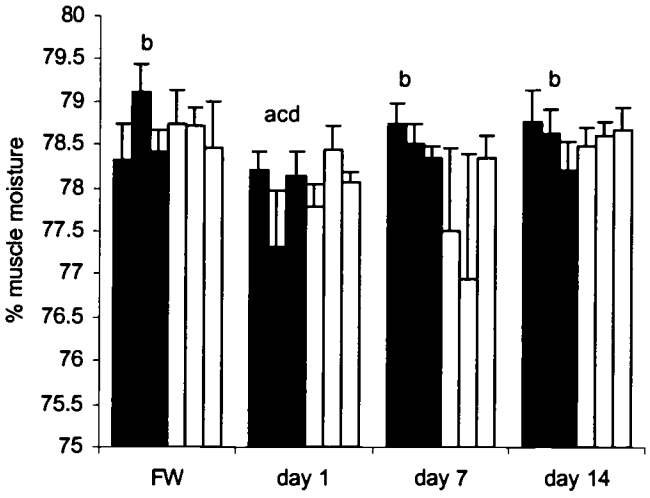


Fig. II.5. Mean (± SE) percent water content of muscle tissue of yearling steelhead immediately after acute stress in FW, and 1, 7, and 14 days after introduction of SW (25 ppt). Grey bars show values for controls and white bars for stressed fish, (n = 8-10, FW: n=5). Indications for significance as shown in Fig. 1.

Hormone levels

Directly after the stressor was applied cortisol levels increased dramatically from basal levels of 7 ng/ml to 150 ng/ml (Fig. II.6 A). At day 1 cortisol of stressed fish declined significantly, whereas two of the control replicates exhibited a rise of cortisol to significantly higher levels than stressed fish on that day or compared to FW control levels. At day 7, cortisol of both groups returned to FW levels, whereas after 14 days a significant increase in comparison to FW and day 7 was noted for control fish. Cortisol in two tanks of the stress treatment was significantly lower than of controls on that day (Fig. II.6 A). Based on the results of the nested ANOVA, differences between replicates for cortisol were not induced by tank effects. Magnesium was positively correlated to plasma cortisol in stressed fish on days 1 and 7 whereas on day 14 a negative relationship between these variables was found. On day 14 potassium and calcium were found to be negatively or positively correlated to cortisol in the

stress treatment, respectively. In controls a positive relation between cortisol and lactate was noted on day 7.

IGF-1 plasma concentrations did not change immediately after applying the stressor but at day 1 after SW exposure IGF-1 of control fish were significantly elevated (Fig. II.6 B). At day 7 both groups experience lower IGF-1 levels than resting levels in FW. IGF-1 concentrations are elevated again at day 14, with significantly higher values in control fish compared to day 7 and to the stressed group (Fig. II.6 B). On days 1 and 7 one tank each of the stress treatment differed statistically from the other replicates, but this did not affect the differences from the data for control fish. This discrepancy between replicates was created by tank effects, however, these effects did not change the interpretation and resultant conclusions of the IGF-1 data. In control fish we found a positive correlation between IGF-1 and length and weight on day 1. Plasma sodium and calcium of controls were positively related to IGF-1 on days 7 and 14. Osmolality, chloride and glucose of control fish showed a positive relationship to IGF-1 on day 14. In the stressed group a positive correlation between IGF-1 and sodium and length was observed in the fish sampled immediately after the stress treatment. Cortisol levels of stressed fish were negatively correlated to IGF-1 on day 1. Osmolality and potassium in stressed fish showed a negative relationship to IGF-1 on day 7. On day 14 we found IGF-1 positively related to lactate in stressed fish.

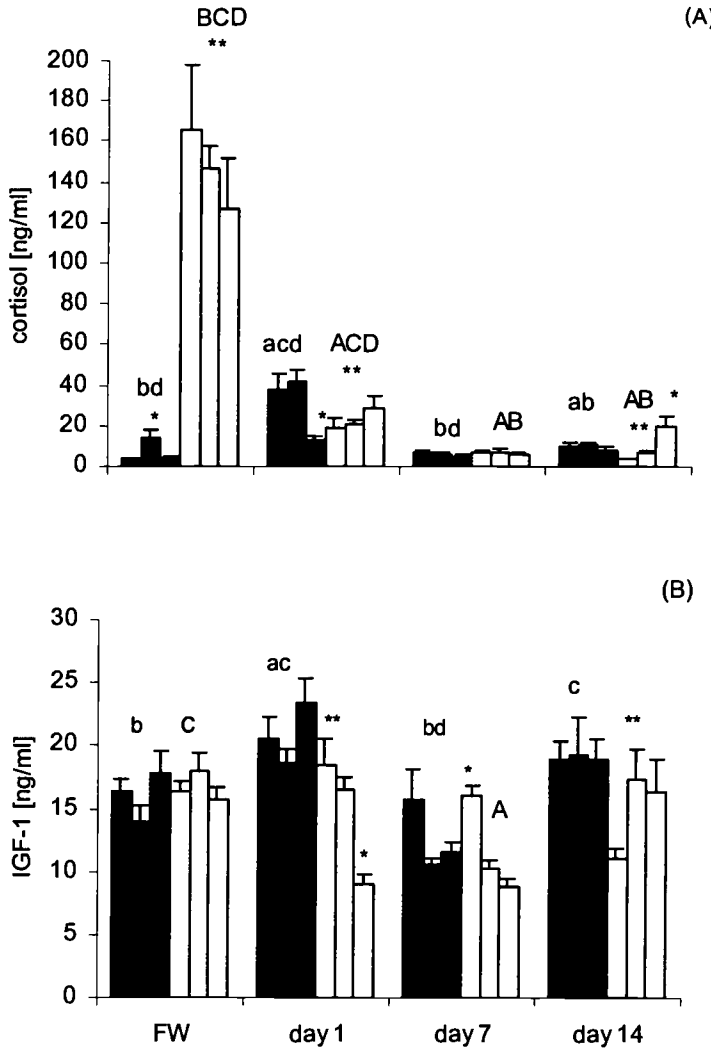


Fig. II.6. Mean (± SE) plasma cortisol (A) and IGF-1 (B) concentrations of yearling steelhead immediately after acute stress in FW, and 1, 7, and 14 days after introduction of SW (25 ppt). Grey bars show values for controls and white bars for stressed fish, (n = 8-10, FW: n=5). Indications for significance as shown in Fig. 1.

Metabolic parameters

The increase of glucose and lactate concentrations after acute stress was highly significant ($p < 0.00005$) (Fig. II.7 A, B). One day after introduction of SW glucose and lactate levels of the stressed group had almost returned to the control levels in FW. Lactate concentrations of both groups remained stable throughout the time in SW and were significantly higher than control levels in FW

(Fig. II.7 A). Glucose levels in control fish steadily increased during the period spent in SW (p = 0), glucose in stressed fish was significantly higher after two weeks compared to day 1 (p = 0) (Fig. II.7 B).

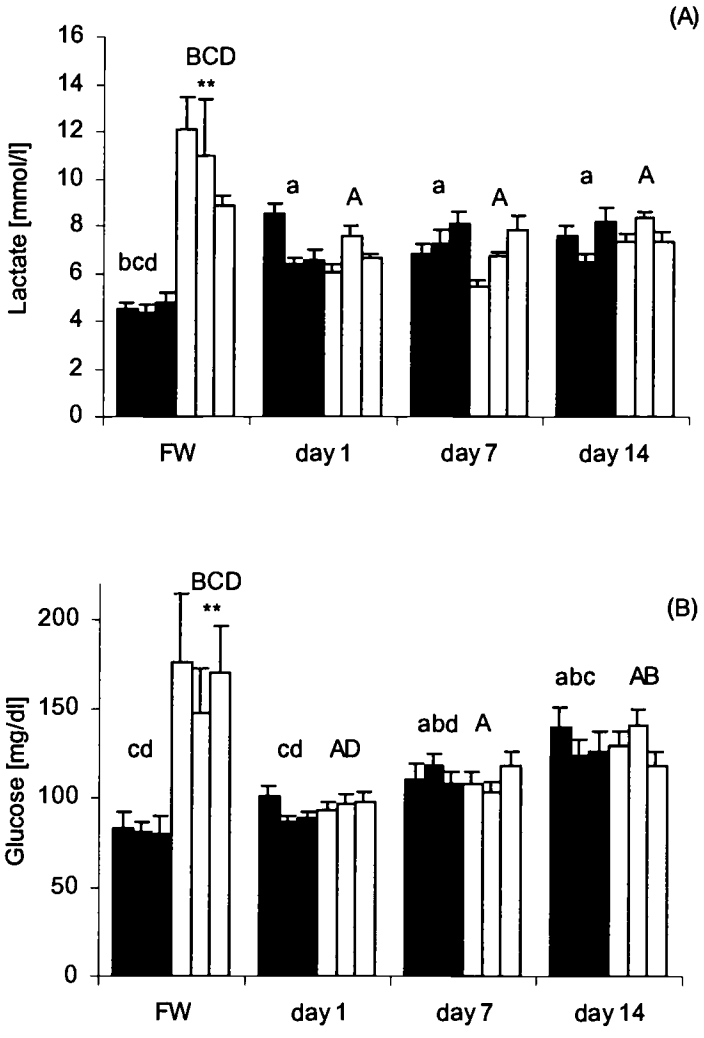


Fig. II.7. Mean (± SE) plasma lactate (A) and glucose (B) concentrations of yearling steelhead immediately after acute stress in FW, and 1, 7, and 14 days after introduction of SW (25 ppt). Grey bars show values for controls and white bars for stressed fish, (n = 8-10, FW: n=5). Indications for significance as shown in Fig. 1.

Discussion

Stress did not significantly decrease feed intake, even though stressed fish showed slightly lower feed intake than controls on day 1. After two weeks stressed fish were eating significantly more compared to day 1. Therefore we infer that a single acute stressor does not impair feed intake during SW acclimation of steelhead. Our data rather suggest that feed intake was suppressed by the change of the media from FW to SW, supported by Pirhonen et al.'s (2003) findings, who observed higher feed intake for steelhead in FW than in SW. The amounts of feed intake we measured were comparable to the results of Pirhonen et al. (2003). We found linear relationships only a few times between individual feed intake and the physiological parameters measured in the present study, suggesting that reduced feed intake was not directly induced by osmoregulatory imbalance. Our data agree with previous studies that reported independent development of SW acclimation from feed intake. Usher et al. (1991) observed suppressed feed intake up to 30 days after SW transfer whereas osmoregulatory homeostasis was reached within 10 days. In our experiment fish were able to osmoregulate within one day spent in SW, using sodium and chloride concentrations as measurements for successful acclimation. Studies with Arctic charr (*Salvelinus alpinus*) showed that osmoregulatory ability was not influenced by nutritional state after transfer to SW (Arnesen et al., 1993, Aas-Hansen et al., 2003).

Our confinement stressor resulted in decreased sodium and chloride levels, which is characteristic for fish exposed to stress while held in FW (Redding and Schreck, 1983). Potassium, magnesium and calcium levels did not respond to the stressor, contrary to studies by Björnsson et al. (1989) and Redding and Schreck (1983) who reported increased concentrations of these electrolytes in

coho salmon (*O. kisutch*) after stress. The occurrence of hypercalcemia and hypermagnesemia after transfer to SW and the subsequent decline of both electrolytes during SW acclimation are in accord with the findings of Björnsson et al. (1989). Sodium and chloride concentrations were slightly but significantly higher on day 1 compared with FW levels. Sodium values were similar to the concentrations for steelhead in FW in Pirhonen at al.'s (2003) study, indicating that both treatments were able to osmoregulate well in the hyperosmotic environment. The only significant differences of electrolytes between stressed and control fish that we found, namely sodium at day 14, potassium at day 7, and calcium at day 1, suggest that stressed fish may have osmoregulated better in SW than controls. Nevertheless, these differences are small and are not consistent between sampling dates. Apart from the control group on day 1, fish were able to maintain the muscle water content at FW levels, again suggesting successful SW adaptation. The lower muscle water content at day 1 of control fish can be explained by the "crisis" period as described by Bath and Eddy (1979), which lasts about 8 h after SW transfer and during which large physiological changes occur. The increase in plasma osmolality in SW is in agreement with higher electrolyte concentrations in SW in comparison to FW.

We did not detect any differences in plasma IGF-1 concentrations immediately after application of the acute stressor. McCormick et al. (1998) observed elevated IGF-1 levels 4 hours after repeated acute stress. A possible increase of total plasma IGF-1 after stress could be masked by elevated IGF-BP levels, which have the same response pattern as cortisol (Kelley et al., 2001). Transfer to SW resulted in increased IGF-1 levels at day 1 and 14 in the control group. McCormick (personal communication, 2003) indicated that unpublished results showed elevated IGF-1 levels in Atlantic salmon after SW exposure for 2

to 14 days. Sakamoto and Hirano (1993) observed increased IGF-1 mRNA levels in gill and kidney after SW transfer. These studies and our results support the hypothesis that IGF-1 is a regulator of hydromineral balance in SW. The differences in IGF-1 levels between stressed and control fish are not reflected in SW adaptability since both groups performed equally well. A possible reason why stressed fish did not show an osmoregulatory response to lower IGF-1 levels could be attributed to the fact that our fish experienced only 25 ppt SW. Another hypothesis is that stressed fish gained an advantage by having higher cortisol levels prior to SW exposure and therefore the increase of IGF-1 was unnecessary or inhibited. The negative relationship between IGF-1 and cortisol on day 1 in stressed fish supports this hypothesis. Positive relationships between IGF-1 and electrolyte levels in controls were observed on days 7 and 14, supporting a possible role for this hormone in osmoregulation. Plasma cortisol levels of the stressed fish were quite high, as typically seen after stress (Redding and Schreck, 1983, Barton and Iwama, 1991). One day after SW transfer cortisol of stressed fish decreased, but both groups had higher levels than control fish in FW, suggesting the involvement of cortisol in osmoregulation in hyperosmotic environments (Redding et al., 1984, Madsen et al., 1995, Wendelaar Bonga, 1997). Another explanation for elevated cortisol levels in controls could be that the animals experienced stress by the switch from FW to SW. The pattern of cortisol and IGF-1 concentrations show some parallels with significantly increased levels of both hormones for controls on day 1 and 14. Possibly IGF-1 and cortisol interact at regulatory pathways, suggested by findings of Young (1988) who observed increased cortisol release after exposure of interrenal tissue to GH.

Elevated glucose levels after stress have been linked to cortisol. The increased glucose levels immediately after stress are likely maintained by

glycogenolysis whereas chronically elevated glucose concentrations are maintained by gluconeogenesis in the liver (Vijayan et al., 1997). The consistently increasing glucose levels after SW transfer suggest higher energy demand for maintenance of the body functions in SW. The gills are the major osmoregulatory sites in SW and are thought to manage energy requirements by oxidation of glucose and lactate (Mommsen, 1984, Soengas et al., 1995). Morgan and Iwama (1991) hypothesized that the lowest energetic costs for osmoregulation are found in the medium in which the species is most commonly found during a particular life stage. Since we used unsmolted fish for the experiment, the costs for osmoregulation should be lower in FW than in SW, explaining the increasing plasma glucose in SW. According to our data cortisol did not seem to be responsible for higher glucose concentrations in SW since cortisol levels decrease during SW adaptation. GH and thyroid hormones, which are known to increase following SW transfer, also have metabolic actions and could be responsible for higher energy demands. Seddiki et al. (1995) found increased oxygen consumption in rainbow trout that were treated with recombinant GH. Increased IGF-1 levels and the positive correlation between IGF-1 and glucose at day 14 in control fish support Seddiki et al.'s (1995) findings but it is unclear, then, why stressed fish had an increase in glucose but not in IGF-1.

The elevation of lactate concentrations immediately after stress is likely due to muscle glycolysis (Moon and Foster, 1995, Suarez and Mommsen, 1987), and lactate may be used for gluconeogenesis in the liver (Vijayan and Moon, 1992). Lactate was significantly increased in SW, which could be caused by higher cortisol concentrations, suggested by the studies of Dugan and Moon (1998) and Laiz-Carrion (2003) who injected rainbow trout and gilthead seabream

(*Sparus aurata*) with cortisol and observed a elevation of lactate similar to our findings.

In summary, feed intake and osmoregulatory ability were not disturbed by a single stressor that was applied before SW transfer. Exposure to SW increased IGF-1 in controls supporting its potential role in osmoregulation; stress likely inhibited the SW transfer-associated elevation of IGF-1. However, both groups were able to osmoregulate well in the hyperosmotic environment. Increasing glucose concentrations and elevated lactate levels indicate higher metabolic costs during SW adaptation.

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EFFECTS OF EXERCISE ON SUBSEQUENT SEAWATER ADAPTATION AND CORTISOL DYNAMICS IN STEELHEAD TROUT (*ONCORHYNCHUS MYKISS*)

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Abstract

The influence of exercise on osmoregulation and cortisol dynamics was examined in juvenile steelhead trout (*Oncorhynchus mykiss*). Plasma cortisol and *in vitro* cortisol secretion by interrenal cells after a 24 hr seawater (SW) challenge test were neither affected by moderate exercise nor by water temperature (13 °C, 21 °C), however, plasma osmolality was lower in exercised fish compared to unexercised fish. Half-life of ^{3}H-cortisol was shorter in fish exposed to exercise whereas metabolic clearance rate did not respond to exercise treatment. Uptake and retention of ^{3}H-corticosteroids in liver and gall bladder were enhanced in exercised fish, and retention of corticosteroids in muscle tissue was longer in unexercised fish. Our findings suggest that exercise likely decreases circulating levels of cortisol in fish and may improve adaptation to SW by enhancing osmoregulatory capability.

Introduction

Cortisol is a key hormone in adaptation to exercise in mammals such as the horse (Lassourd et al., 1996). Cashmore et al. (1977) and Dybdal et al. (1980) reported increased plasma cortisol levels after exercise in man and horse, but its role in exercise of fishes is largely unknown. Salmonids had initially elevated cortisol concentrations in response to exercise (Nielsen et al., 2000, Boesgaard et al., 1993), however, after exercising for 24 hr lower plasma cortisol levels were observed compared to controls in still water (Boesgaard et al., 1993). Cortisol, the major corticosteroid in most teleostean fishes (Chester Jones et al. 1969), is a primary messenger of the stress response in fish (Schreck, 1981) and is also important for SW acclimation (McCormick, 1995). Wendt and Saunders (1973)

found a faster reduction of high blood lactate levels following exercise, and a better adult recapture rate for exercise trained Atlantic salmon (*Salmo salar)*.

Examining plasma hormone levels will provide limited information about hormone dynamics such as secretion rate and metabolic clearance rate (MCR), which are equally important parameters of hormone dynamics. MCR is defined as the volume of plasma, which is irreversibly cleared of hormone per unit of time (Tait and Burstein, 1964), and it is dependent upon binding proteins, target tissue receptors, tissue uptake and catabolism of cortisol (Mommsen, et al. 1999). Little is known about the influence of exercise on secretion or clearance of cortisol in fish. In horses it has been shown that an increase of cortisol secretion rate in exercised animals was not reflected likewise in plasma cortisol levels because MCR of cortisol was largely increased (Lassourd et al., 1996).

Several studies on salmonids reported increased osmoregulatory ability in seawater (SW) after exercise (Gallaugher et al., 2001, Khovanskiy et al.,1993), whereas Jørgensen and Jobling (1993, 1994) did not find an improvement in SW adaptation after exercise training. Higher energy demands during exercise, however, may deplete energy stores in migrating juvenile salmonids (*Oncorhynchus. spp.*) (Congleton et al., 1998, Beckman et al., 2000) and as a consequence reduce the ability to adapt to SW. Water temperature is an important environmental factor influencing physiological functions such as growth, swimming performance, cardiac output, and metabolic rate of fishes (Beamish, 1978, Farrell, 1984).

The aim of this study was to characterize the influence of exercise on SW adaptation, to determine the effects of exercise on corticosteroid dynamics, and determine if temperature affects these processes in juvenile steelhead.

Materials and methods

Experimental animals

Juvenile steelhead trout obtained from the Alsea hatchery (Oregon Department of Fish and Wildlife) were held at 12 – 13 °C, in flow-through tanks at Oregon State University's Fish Performance and Genetics Laboratory fed Bio-Oregon semi-moist pellets at 2% body weight per day.

Experimental design – Experiment 1: Effects of exercise on cortisol secretion

On September 27, 2002 fish (FL = 115 ± 1.144 mm, weight = 15.3 ± 0.451 g) were transferred from the stock tank to 1 m diameter circular, flow-through tanks. Groups of fish (n=12) were randomly assigned to 12 tanks, and all treatment groups were triplicated. After a one-week acclimation period water temperature was increased stepwise in six of the tanks from 13 °C to 21 °C over a two-week period by adjusting the flow of heated and cold water. The temperature for the cold-water treatment was 13 °C. Flow was kept equal for heated and cold-water tanks with a final flow rate of 2 l/min. A circular current was provided in three cool and three warm tanks by a pump outside of the tanks (CustomSeaLife, Inc.), which pumped water into the tank over spray bars yielding a velocity of approximately 1.7 body length/sec (BLS). Fish in these tanks were thus required to swim to maintain position. In the remaining tanks water was introduced at a 90° angle to the water surface, therefore no water current was generated, and the fish in those tanks experienced essentially no directional current.

Fish were fed twice a day at maintenance level, taking into account energy expenditures at different water temperatures and swimming speeds. The

treatment was stopped after five weeks and a 24 h SW challenge test was performed with all treatment groups on November 18, 2002. The general procedures followed those from Blackburn and Clarke (1987). Saltwater (Instant Ocean®) was introduced as a brine into tanks reaching a final concentration of 21 ppt, the salinity that was determined in a preliminary experiment resulting in 100% survival. Water flow was discontinued in all tanks and aeration was provided by air pumps. Fish were not fed during the SW exposure. Two fish per tank were sampled before the SW challenge test; the remaining fish were sampled 24 h after introducing the SW.

Sampling procedures

Fish were netted and killed with a lethal dose of tricaine methansulfonate (MS-222, 200 mg/l, buffered with $NaHCO_3$), and bled into heparinized capillary tubes by severing the caudal peduncle. Plasma was separated by centrifugation, frozen and stored at –80 °C until analysis. If necessary, equal amounts of plasma from several fish from the same tank were pooled for analysis of plasma cortisol osmolality. This resulted in no more than two pooled samples per tank.

Head kidney incubation

Methods developed by Patiño et al. (1986b) were followed to determine cortisol secretion rates. The head kidney of each fish was excised and placed into separate tissue culture wells (24 well plates) containing 1.5 ml ice-cold of Leibovitz medium. Head kidney tissue was minced into about 1 mm^3 fragments and transferred to new wells with fresh medium. Plates were then placed on an orbital shaker at 100 rpm and pre-incubated at 14 °C for about 4 hr, with a

medium change after 2 hr. Tissues were then transferred to new plates and incubated with either hormone-free medium or medium containing 50 mU/ml porcine ACTH (Sigma). After 3 hr the incubation medium was removed and frozen at –80 °C until further analysis.

Plasma and incubation media analysis

Levels of cortisol in incubation media (50 μl) and plasma (10 μl) were determined by radioimmunosassay following the method of Foster and Dunn (1974), as modified by Redding et al. (1984a). All fish which had less than 3.9 ng/ml of cortisol (the lowest detectable standard in the assay) were assigned this value. Plasma osmolality was measured with a vapor pressure osmometer.

Experimental design - Experiment 2: Effects of exercise on cortisol clearance

Fish (FL = 240 ± 2.626 mm, weight = 141.4 ± 3.934 g) were transferred on May 12, 2003 to 1m diameter circular, flow-through tanks. Treatments were duplicated with 27 fish per tank. Before fish were randomly assigned to the tanks, they were anesthetized to determine length and weight. Fish were fed twice daily at maintenance level (1% of bodyweight). After this acclimation period the treatment was started on June 2, 2003. The water was introduced through spray bars into the tanks yielding a circular current at 1.3 BLS for the exercise treatment. Tanks for the unexercised group were equivalent to those from experiment 1.

^{3}H-cortisol administration and blood sampling

Methods to determine clearance of cortisol essentially followed the methods developed by Redding et al. (1984b). Three fish from each tank were sampled before the others were injected with ^{3}H-cortisol. For the administration of ^{3}H-cortisol fish were anesthetized with 50 mg/l MS-222 buffered with 125 mg/l $NaHCO_3$ and injected intracardially with 5 μCi of [1,2,6,7- ^{3}H]cortisol (Perkin Elmer) using a glass syringe (Hamilton). ^{3}H-cortisol was delivered in 100 μl of 5% ethanol:saline solution. After recovering, the fish were transferred back into the treatment tanks. Injection of all 25 fish from one tank took approximately 20 min. At 0.25, 0.5, 1, 4, 12, and 24 hr after injection fish were netted and killed with a lethal dose of MS-222. Fish were weighed (to 0.1 g) and measured for fork length (to 1 mm). Blood samples were taken from the caudal peduncle using heparinized vacutainers (Becton Dickinson) and immediately centrifuged. 20 μl of plasma were solubilized with 200 μl Optisolv (Perkin Elmer) in glass scintillation vials.

Approximately 100 mg of tissue sample was taken from liver, muscle, gill filament, kidney and the entire gall bladder including the bile at 4, 12, and 24 hr after injection. The tissues were placed into pre-weighed scintillation vials, weighed and 1 ml Optisolv was added. After tissues and plasma were completely solubilized, 10 ml scintillation fluid was added and total radioactivity was determined with a liquid scintillation counter. The counts of the tissues were corrected for quenching effects.

Calculation of MCR and half-life

The MCR of total-^{3}H is inversely proportional to the area (A) under the curve of total-^{3}H versus time, where MCR = 100/A when radioactivity is expressed as a percentage of the injected dose per ml plasma. Area was calculated by the

trapezoidal rule (Normand and Fortier, 1970). $T_{1/2}$ was determined using the equation: $T_{1/2} = - (\ln 2/m)$, where m is the slope of concentration of total-^{3}H versus time. Values for both radioactivity, and time were log-transformed.

Statistical analyses

For experiment 1 differences between replicate tanks within treatment and between treatments were analyzed using a one-way analysis of variance (ANOVA), followed by a Tukey-Kramer test if there was a significant effect. For experiment 2 differences within and between treatments were analyzed using a Welch two-sample t-test. If no differences within the treatments were detected replicate tanks were pooled.

Results

Experiment 1

Osmolality increased from 301 mmol/kg in FW to 322-344 mmol/kg after the 24 hr SW challenge (data not shown). Exercised fish experienced significantly lower plasma osmolalities than unexercised fish (Fig. III.1). Since one tank of the exercise treatment group at 21 °C was significantly different from the other two triplicates, non-pooled results are presented. Osmolality of fish acclimated to 21 °C was not significantly different compared to fish held at 13 °C.

Plasma cortisol levels after the SW challenge test showed no statistical differences between treatments with mean values between 12.2 and 16.3 ng/ml (Fig. III.2). Cortisol levels before the SW challenge were higher than after the SW challenge but because of the small sample size of two fish per tank and high individual variability we could not statistically compare these values (data not

shown). Neither exercise nor temperature had an effect on resting or ACTH-stimulated *in vitro* cortisol release by the interrenal cells (Fig. III.3). Exercised fish had a significantly higher condition factor than unexercised fish (Fig. III.4).

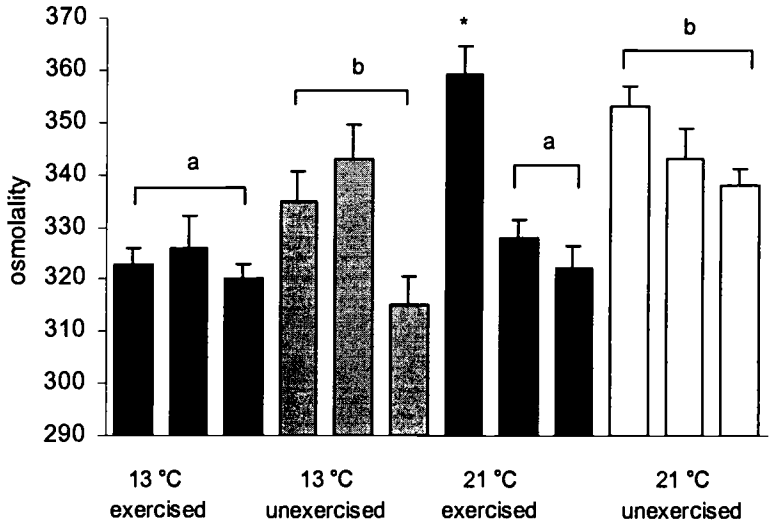


Fig. III.1. Mean (± SE) plasma osmolality of exercised and unexercised subyearling steelhead (in triplicate) after a 24 hr SW challenge test (21 ppt). Each bar represents 6-10 samples.* = denotes significantly * = significantly different ($p < 0.05$) from other tanks within replicates; bars with the same letter for a replicate were not significantly different ($p > 0.05$).

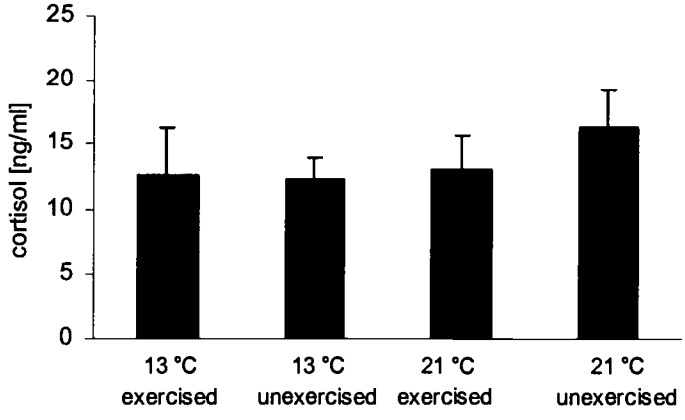


Fig. III.2. Mean (± SE) plasma cortisol of exercised and unexercised subyearling steelhead after a 24 hr SW challenge test (21 ppt). Each bar represents 19-25 samples.

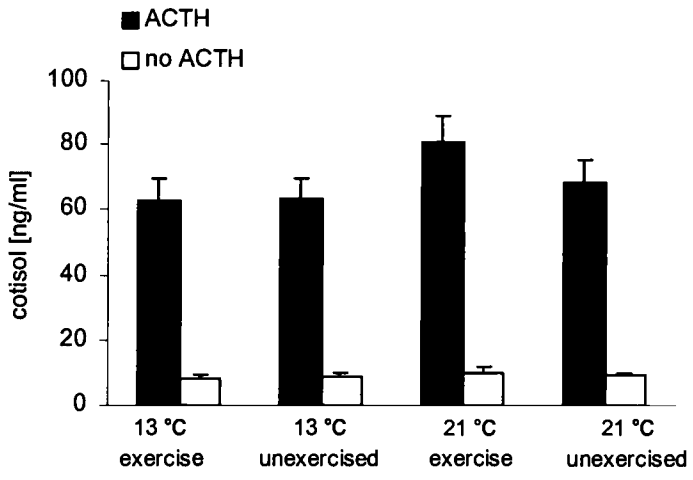


Fig. III.3. Mean (± SE) cortisol concentration in incubation medium with interrenal tissue of exercised and unexercised subyearling steelhead after a 24 hr SW challenge test (21 ppt). Each bar represents 15 fish. Grey bars show values for tissues exposed to 50 mU/ml porcine ACTH and white bars for tissues that were not exposed to ACTH.

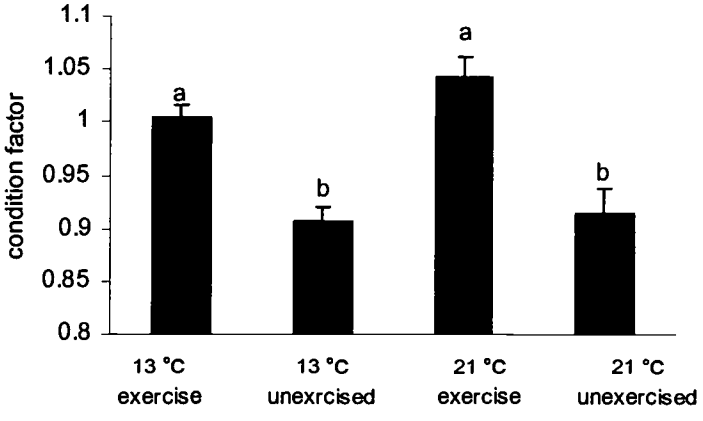


Fig. III.4. Mean (± SE) condition factor of exercised and control subyearling steelhead. Each bar represents 36 fish. Bars with the same letter were not significantly different ($p > 0.05$).

Experiment 2

The amount of total-^{3}H present in the tissues after injection with ^{3}H-cortisol decreased with time in plasma (Fig. III.5), gill, kidney, muscle, and liver and increased over time in the gall bladder of both treatments (Fig. III.6). Total-^{3}H in plasma was significantly higher at 0.25 hr and significantly lower at 24 hr post injection in exercised fish. Total-^{3}H of muscle was significantly higher at 12 hr in unexercised fish. Total-^{3}H in liver at 24 hr was significantly higher in unexercised compared to exercised fish. Tissue-plasma ratios of total-^{3}H increased for all tissues with time after injection. The ratios for liver and gall bladder at 12 hr were significantly higher in the exercised group. Fish that exercised had a significantly shorter $T_{1/2}$ of total-^{3}H-cortisol and metabolites than the fish that did not exercise. The MCR of total-^{3}H did not differ between the treatments (Table 1). The condition factor in exercised fish was significantly higher than for unexercised fish.

Table 1. Mean (± SE) condition factor, and metabolic clearance rate (ml/kg/hr), and half-life (hr) of ^{3}H-cortisol in exercised and unexercised yearling steelhead

Treatment	*Condition factor*	*Half-life*	*MCR*
Exercise	1.02 ± 0.016[a]	2.29 ± 0.072[a]	11.86 ± 0.836
Non-exercise	0.96 ± 0.017	2.56 ± 0.069	10.93 ± 0.686

[a] denotes significantly different from non-exercised fish ($p < 0.05$)

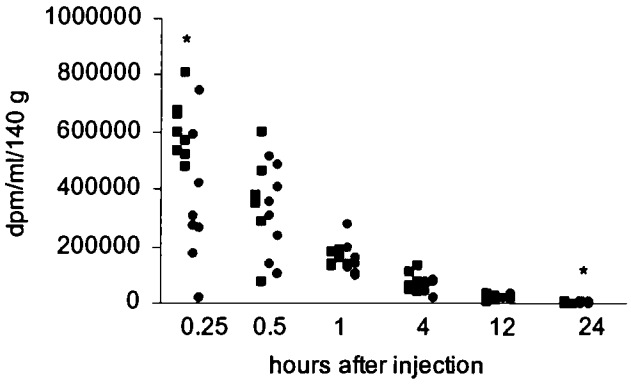
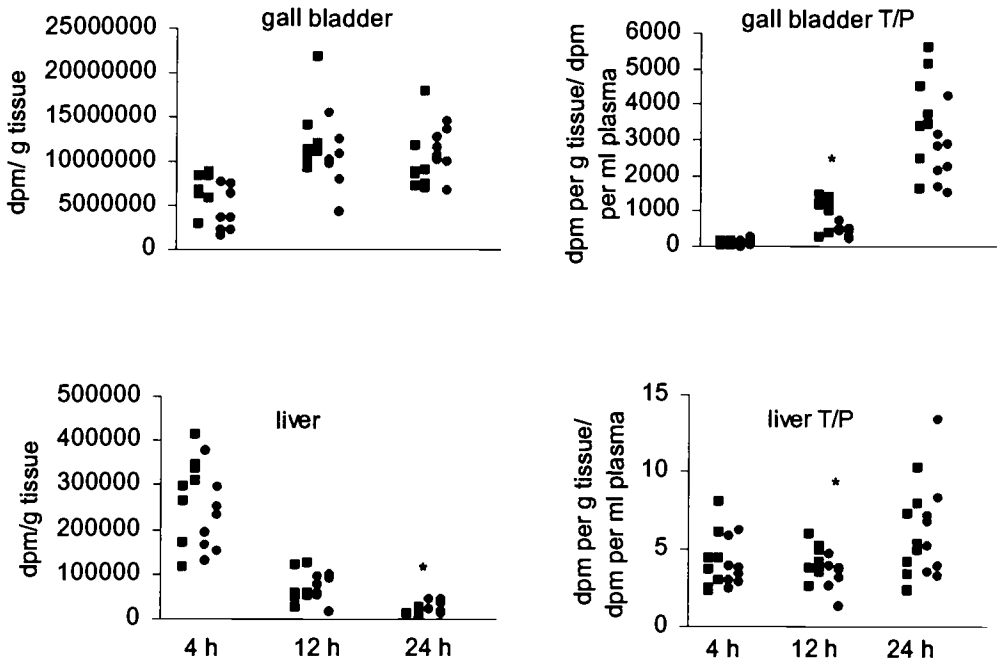


Fig. III.5. Total ^{3}H-radioactivity of plasma versus time in exercised and unexercised yearling steelhead after injection of ^{3}H-cortisol. Each symbol represents the value for one fish, squares show values for exercised, circles for unexercised fish and symbols that are in the same column represent fish from the same tank. * = denotes treatments that are significantly different ($p < 0.05$)



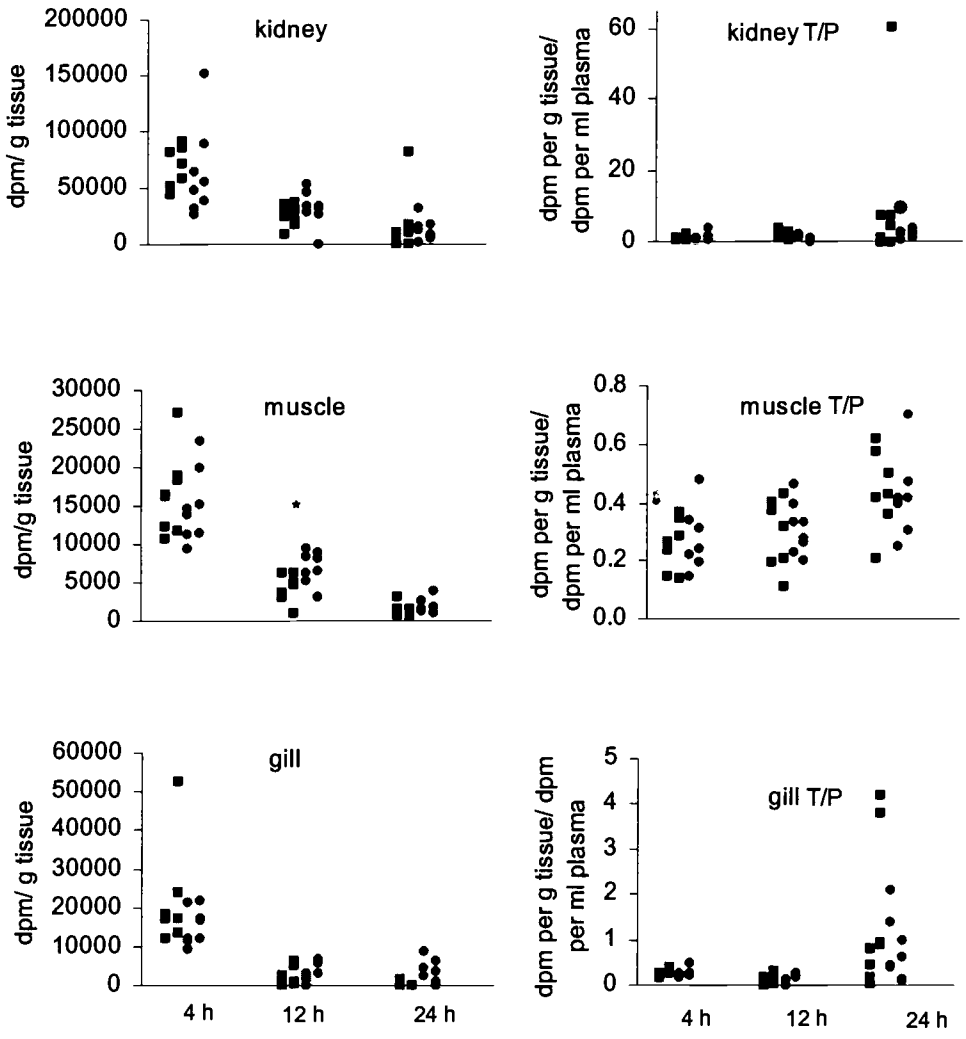


Fig. III.6. Total ^{3}H-radioactivity and tissue/plasma ratio (T/P) of tissues at 4, 12, and 24 hr after injection of ^{3}H-cortisol in exercised and unexercised yearling steelhead. Each symbol represents the value for one fish, squares show values for exercised, circles for unexercised fish and symbols that are in the same column represent fish from the same tank. * = denotes treatments that are significantly different ($p < 0.05$)

Discussion

Plasma cortisol and *in vitro* cortisol secretion levels were neither affected by exercise treatment nor by temperature, and plasma cortisol of all treatments was relatively low after the 24 hr SW challenge test, suggesting that the

introduction of SW was either unstressful or only minimally stressful. Nielsen et al. (1994, 2000) observed in rainbow trout an initial cortisol increase at the onset of exercise followed by a return to basal levels within 24 hr. Boesgaard et al. (1993) found lower cortisol levels in Atlantic salmon that were exposed to moderate exercise for 24 hr compared to fish in standing water. In our study neither exercise nor temperature affected cortisol levels. Van Ham et al. (2003) reported more rapid cortisol increases in turbot (*Scophthalmus maximus*) after exercise at 18 and 22 °C compared to 10 °C, perhaps resulting from a more rapid cortisol release consequent to a higher metabolic rate (Sumpter et al. 1985).

Plasma osmolality clearly increased after SW transfer, and values for unexercised fish were significantly higher than for exercised fish. Plasma osmolalities in fish from the first experiment are similar to those reported by Jørgensen and Jobling (1993) for Atlantic salmon smolts after exercise followed by a 24 hr SW challenge test. Our results also agree with the findings of Khovanskiy et al. (1993) who reported that exercise increased the ionoregulatory capability of juvenile chum salmon (*O.* keta) after transfer to SW. Gallaugher et al. (2001) observed lower plasma osmolality in exercise-trained chinook salmon (*O. tshawytscha*) during swimming at critical speed and proposed that higher oxygen consumption in exercise-trained fish provided benefits to other systems such as the osmoregulatory system. The enhanced osmoregulatory ability in exercise-trained fish may be due to increased gut blood flow during exercise resulting in greater intestinal water absorption (Thorarensen et al., 1993). Our results suggest that exercise at an aerobic level in fresh water improves the adaptation of juvenile steelhead to SW, and if applied in an aquaculture setting could lead to higher ocean survival. Temperature did not have a significant effect on SW adaptation. Our results imply that fish exposed to higher temperatures during rearing or

migration do not have a reduced ability to osmoregulate in the ocean environment.

We also found that exercised fish had a shorter $T_{1/2}$ of total-^{3}H than non-exercised fish. Total-^{3}H in plasma at 24 hr was significantly lower in exercised fish, supporting our finding of a shorter $T_{1/2}$ for corticosteroids in this group of fish. Decreased half-life due to exercise has also been observed in mammals (Few, 1974, Lassourd et al., 1996). Given the shorter $T_{1/2}$ we would have expected to see a higher MCR in exercised fish, however there were no differences in MCR between the treatments. It is possible that the differences we detected for $T_{1/2}$ were too small to be evidenced in the calculation for corresponding MCR. The MCR for cortisol observed in our study was considerably lower than those found in other studies that ranged between 45 ml/kg/h for American eel (*Anguilla rostrata*) to 270 ml/kg/h for sockeye salmon (*O. nerka*) (Butler, 1973, Donaldson and Fagerlund, 1970). The MCR that we found was also lower than that determined by Redding et al. (1984b) for coho salmon (*O. kisutch*), who used the same methodology as our study. This discrepancy could be attributed to species differences and/or differences in life history stage and environmental factors.

Total-^{3}H in the liver decreased with time whereas the tissue/plasma ratio increased. Total-^{3}H was higher for the unexercised group at 24 h, and the tissue/plasma ratio was greater at 12 h for exercised fish. These results suggest that uptake and retention of cortiscosteroids occurred in the liver, and that this process was enhanced in exercised fish. Both, radioactivity and tissue/plasma ratios in the gall bladder increased over time, confirming that bile is the major route for excretion of metabolized cortiscosteroids (Idler and Truscott, 1972, Redding et al., 1984b). Higher tissue/plasma ratios at 12 hr in the gall bladder of exercised fish support our finding of a shorter $T_{1/2}$ of corticosteroids for this group.

Total-^{3}H in muscle, gill, and kidney decreased over time, but tissue/plasma ratios increased. Radioactivity in muscle tissue at 12 hr was higher in the unexercised fish, suggesting that the retention of total-^{3}H was longer than in exercised fish. Exercise increases muscle blood flow in mammals (Parks and Manohar, 1985), and exercise training is known to improve capillarity in fish muscles (Davie et al., 1986, Sänger, 1992). This could explain the faster excretion of total-^{3}H from the muscle tissue in exercised fish from our study. Farrell et al. (1990, 1991) found that exercise-trained rainbow trout had a higher cardiac output, a larger stroke volume, and higher levels of cardiac enzymes. We assume that the enhanced cardiac performance of exercised fish in our experiment led to a quicker removal of total-^{3}H from the plasma and a faster uptake of total-^{3}H in the gall bladder. The faster removal of total-^{3}H from the plasma suggests that exercise can reduce levels of circulating corticosteroids.

Based on the shorter $T_{1/2}$ of corticosteroids found in our second experiment we would have expected to find either lower plasma cortisol levels or increased cortisol secretion rates in the exercised fish in our first experiment. This disagreement is difficult to explain but could be due to seasonal effects or differences in fish size.

The condition factor of the exercised fish was higher than for unexercised fish in both experiments, agreeing with the findings of Jørgensen and Jobling (1993, 1994). An increase in mesenteric fat, as observed by Nielsen et al. (2000), may have contributed to the increased condition factor of the exercised group.

In summary, moderate exercise of juvenile steelhead in FW improved osmoregulatory capability during the subsequent SW challenge test, decreased $T_{1/2}$ of ^{3}H-cortisol, and enhanced uptake of corticosteroids in the liver and gall bladder. The clearance of ^{3}H-cortisol from the muscle tissue occurred faster in

exercised fish. These findings present positive potential for using exercise training in aquaculture, which could lead to a higher ocean survival of juvenile salmonids. We further speculate that exercise-trained fish can recover faster from stress due to the quicker removal of corticosteroids from the plasma.

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CHAPTER IV

GENERAL CONCLUSION

The goal of this thesis was to determine the effects of stress and moderate exercise on following SW adaptation in juvenile steelhead. The results of chapter II suggest that a single stressor applied directly before transition from FW to SW has no effect on feed intake and osmoregulatory ability of juvenile steelhead in SW. Our results implicate a potential role of IGF-1 in stress response and SW adaptation. IGF-1 levels were increased after SW transition in control fish. This response, however, did not occur after SW transition for fish that had first been exposed to stress. Nevertheless, stressed fish were able to osmoregulate equally well indicating that other factors besides cortisol and IGF-1 promoted osmoregulation of stressed fish in SW. Increasing glucose levels in stressed and control fish during SW acclimation suggest higher metabolic costs in SW, probably to provide energy for ion pumps in the gills.

In chapter III we found that exercise reduces half-life of total-^{3}H, suggesting the faster removal of cortisol from the plasma of exercised fish compared to unexercised fish. This finding suggests that exercise could help moderate the physiological response to stress in fish. We also observed enhanced osmoregulatory ability in SW in exercised fish. Both findings indicate that exercise treatment is beneficial for juvenile steelhead and if applied in aquaculture may have positive effects on SW acclimation and therefore lead to a higher ocean survival. Likely, these beneficial effects are due to increased cardiac performance after exercise treatment, which allows the faster removal of cortisol from plasma and uptake by the gall bladder. Surprisingly, there was no effect on

osmoregulatory ability and cortisol levels due to water temperatures indicating that the higher temperature was not stressful for the fish. I would have expected to see higher cortisol levels or impaired osmoregulation in SW in the fish acclimated to 21° C because this temperature is clearly above the temperature optimum for steelhead.

In summary, this research provides information on how acute stress affects SW acclimation in juvenile steelhead focusing specifically on the role of cortisol and IFG-1. Furthermore, we found that exercise prior to SW entry provides benefits for osmoregulation in SW and reduces circulating corticosteroid levels in these fish.

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APENDIX

EFFECTS OF REPEATED STRESS ON OSMOREGULATION IN JUVENILE STEELHEAD TROUT (*ONCORHYNCHUS MYKISS*)

The goal of this study was to determine the effects of a repeated confinement stressor on seawater (SW) adaptation in juvenile steelhead (*Oncorhynchus mykiss*).

Materials and methods

Juvenile Steelhead trout were obtained from the Alsea hatchery (Oregon Department of Fish and Wildlife). Fish were held at 12 - 13° C, in flow-through tanks at Oregon State University's Fish Performance and Genetics Laboratory and were fed Bio-Oregon semi-moist pellets at ~ 2% body weight per day. In mid-August fish were transferred from the stock tank to ~ 1 m diameter circular tanks, 12 fish per tank; all treatment groups were triplicated. After a 14-day acclimation period a stress treatment was applied for two weeks, twice per day. The stressor consisted of netting the fish in a perforated 18-liter bucket, and keeping the fish confined for one hour in the bucket immersed in the tank. A control group of fish did not undergo the stress treatment. A 24 hr SW challenge test (21 ppt) was performed with all fish at the following day after completion of the stress treatment at September 9, 2002. After 24 hr fish were netted and killed with a lethal dose of tricaine methansulfonate (MS222, 200 mg/l, buffered with $NaHCO_3$), and bled into heparinized capillary tubes by severing the caudal peduncle. 12 fish from the same stock that did not experience the SW challenge test were sampled for comparison. Blood plasma was separated by centrifugation, frozen and stored at – 80° C until analysis. If it was necessary equal amounts of plasma from several fish of the same tank was pooled for analysis. Plasma cortisol levels were

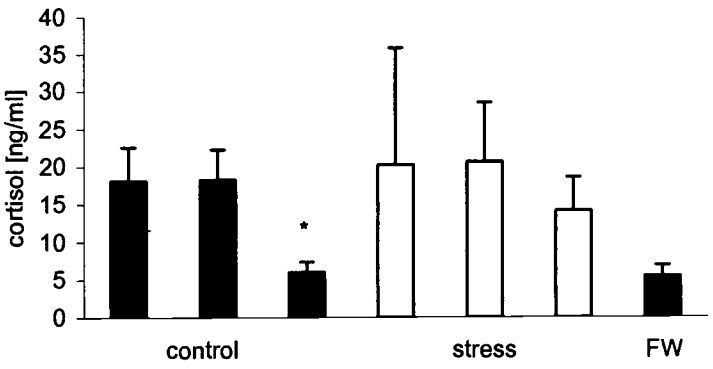
determined by radioimmunosassay following the method of Foster and Dunn (1974) modified by Redding et al. (1984). Plasma osmolality was measured with a vapor pressure osmometer.

Statistical tests were performed with S-Plus 6.1. Differences between replicate tanks within treatment were analyzed using one-way analysis of variance (ANOVA). Differences between treatments were analyzed using a two-sample t-test. If no differences within the treatments occurred replicate tanks were pooled.

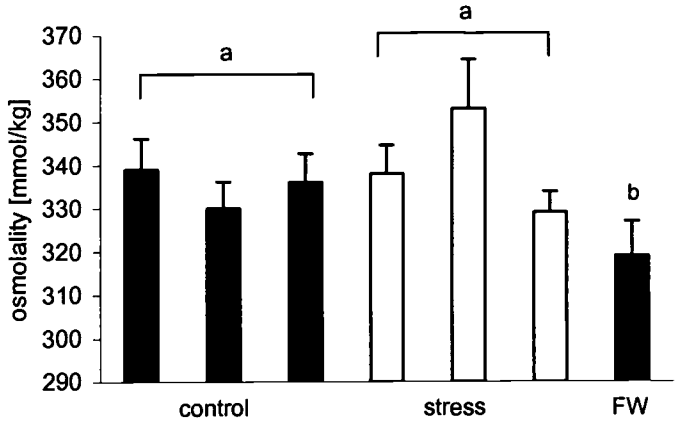
Results

Plasma cortisol levels of fish from two of the control tanks were not different from the stressed fish. One of the control tanks had fish with significantly lower levels compared to the other two tanks of control fish and to stressed fish (Fig. 1). Plasma osmolality after the 24 hr SW challenge test did not differ between stressed and control fish (Fig. 1), but was significantly higher in SW than for fish from the same stock that did not experience the SW challenge test ($p < 0.05$).

In summary, repeated confinement stress for two weeks did not impair subsequent SW adaptation, and plasma cortisol levels were not affected by the stress treatment.



Appendix
Fig. 1. Mean (± SE) plasma cortisol triplicated of repeatedly stressed and control subyearling steelhead after a 24 hr SW challenge test (21 ppt), and fish from the same stock that were not exposed to SW (n = 12 fish, partly pooled). *, significantly different ($p < 0.05$) from other tanks within replicate.



Appendix
Fig. 2. Mean (± SE) plasma osmolality triplicated of repeatedly stressed and control subyearling steelhead after a 24 hr SW challenge test (21 ppt), and fish from the same stock that were not exposed to SW (n = 12 fish, partly pooled). Bars with the same letter are not significantly different ($p > 0.05$).

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